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Ramichloridium apiculatum, a new record for China, causing sooty blotch and flyspeck

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Abstract — *Ramichloridium apiculatum* associated with sooty blotch and flyspeck is described and reported for the first time in China. The strains were isolated from the cuticle of pear and apple collected from orchards in Anhui and Jiangsu Provinces. Morphological characters and phylogenetic analysis, based on ITS sequences, indicated that the fungus is distinct from the other known species in the genus *Ramichloridium*.

Key words — *Dissoconiaceae*, *Capnodiales*, discrete speck, taxonomy, phylogeny

Introduction

Sooty blotch and flyspeck (SBFS) is a fungal complex that causes blemishes on the epicuticular wax layer of a variety of plants. It occurs in humid regions worldwide and is regarded as an economically serious disease (Batzer et al. 2008, Gleason et al. 2011, Li et al. 2012).

Ramichloridium was originally described by Stahel (1937), but the name was invalid because of the lack of a Latin diagnosis. The genus was validated by de Hoog (1977) with *Chloridium apiculatum* (*R. apiculatum*) as type species. The generic characters used for taxonomic circumscription by de Hoog (1977) were erect dark branched or non-branched conidiophores with different degrees of differentiation, sympodial proliferation, and aseptate conidia (Arzanlou et al. 2007). According to MycoBank (accessed March 30, 2014), more than 39 species of *Ramichloridium* have been proposed.

The objective of this paper is to identify and describe *Ramichloridium apiculatum* as a newly recorded causal agent of SBFS on pear and apple from China.

Materials & methods

Isolates and morphology

Strains were isolated from colonies displaying the discrete speck (DS) mycelial type (Batzer et al. 2005), LWHDSL1 (GenBank KC986369), LWHDSL5 (GenBank KC986370), LWHDSL26 (GenBank KC986371) were collected by WH Li on pear from orchards near Dangshang, Anhui Province and WLXZSN33 (GenBank KC986372), WLXZSN43 (GenBank KC986373) were collected by L Wang & YM Zhao on apple in Xuzhou, Jiangsu Province in China during October 2012. Individual sclerotium-like bodies (Batzer et al. 2005) were transferred from the surface of pear and apple to slants of potato-dextrose agar (PDA, Difco) and cultured at 25°C in the dark (Sun et al. 2003). After about 1 month, colonies were transferred to fresh PDA and malt extract agar (MEA, Difco) plates for describing and photographing. Additionally, hyphal tips from PDA slants were transferred to fresh PDA plates into which a sterile cover slip had been partially inserted into the agar at a 60° angle adjacent to the inoculum, in order to induce the fungus to grow onto the cover slip. Fungal fruiting structures that formed on the cover slips were examined, and 30 measurements were made of each feature.

Culture characteristics were described based on colonies on PDA plates at 25°C for one month and on MEA plates at 24°C for 14 d, both of which were incubated in darkness. Specimens and cultures were deposited in Fungal Laboratory, Northwest A&F University.

DNA extraction, PCR, and sequencing

The fungus was grown on PDA and genomic DNA was extracted following Li et al. (2011). The primer pairs ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) were used to amplify and sequence the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. The PCR reaction consisted of 1 unit of Taq polymerase, 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, and 2 µL of template DNA, made up to a total volume of 25 µL with sterile water. Amplifications were performed on a Bio-Rad PCR System S1000TM Thermal Cycler. The PCR cycling parameters were initial denaturation at 94°C for 90 min, 35 cycles of denaturation at 94°C for 35 s, annealing at 52°C for 60 s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. Sequencing of the PCR product was performed at Sangon Biotech (Shanghai, China).

Sequence alignment and phylogenetic analysis

All ITS nucleotide sequences were aligned with GenBank sequences that displayed a high degree of similarity. All related sequences and the outgroup *Mycosphaerella marksii* were imported into CLUSTAL-X (Thompson et al. 1997). Preliminary alignments were manually adjusted using BioEdit 5.0.9.1 (Hall 1999) where necessary.

The aligned DNA sequences underwent maximum parsimony analysis with PAUP vers. 4.0b (Swofford 2003). Heuristic searches were conducted with 1,000 random taxa addition. A majority consensus tree was constructed and clade stability was assessed by 1,000 bootstrap replications. Other measures were also calculated for parsimony, including tree length, consistency index, retention index, and rescaled consistency index (CI, RI and RC). The ITS sequences generated in this study were deposited in GenBank as accessions KC986369–986373.

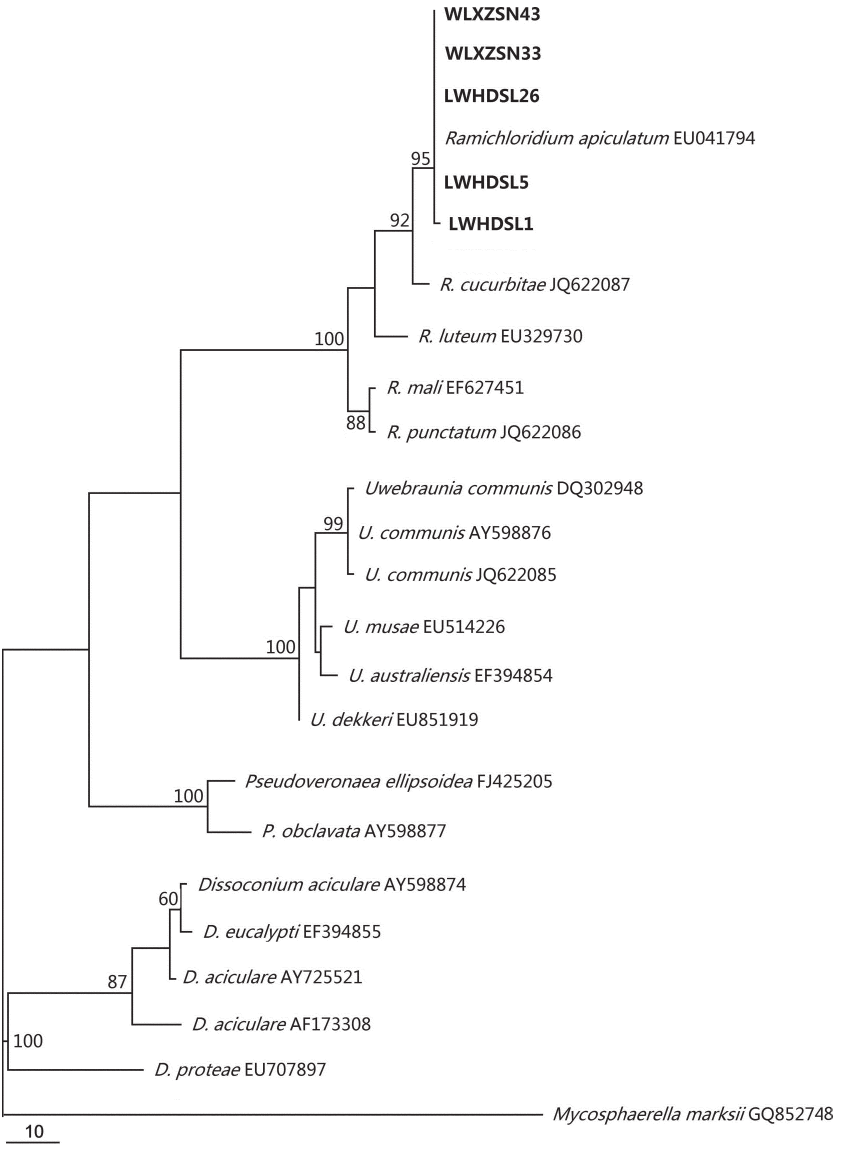

Fig. 1. One of 100 equally most parsimonious trees determined by ITS sequence (TL = 309 steps, CI = 0.7961, HI = 0.2039, RI = 0.9088, RC = 0.7235). Bootstrap support values (>50%) based on 1000 replicates are shown at the nodes. The scale bar denotes 10 changes. The tree is rooted to *Mycosphaerella marksii* and strains treated in this study are shown in bold.

Results

Phylogenetic analysis

The rDNA-ITS alignment for 24 taxa including the outgroup comprised 499 total characters, of which 322 were constant, 66 were parsimony-uninformative, and 111 were parsimony-informative; gaps were treated as "missing." One of 100 most parsimonious trees that were generated is shown in Fig. 1. *Ramichloridium* species separated into independent clades with well-supported bootstrap values. Our five strains clustered together with *R. apiculatum* to form a sub-clade with a bootstrap value of 95%, suggesting that these strains are conspecific with *R. apiculatum* (Fig. 1).

Taxonomy

Ramichloridium apiculatum (J.H. Mill., Giddens & A.A. Foster) de Hoog, Stud. Mycol. 15: 69, 1977 Fig. 2

Mycelium hyphae smooth, hyaline to subhyaline, thin-walled, 1.5–2.0 µm diam. Conidiophores erect, arising at right angles from superficial hyphae, unbranched, 1–3-septate, thick-walled, dark brown, ≤75 µm long. Conidiogenous cells integrated, terminal, subcylindrical, golden-brown, thick-walled, smooth, (23–)25–40(–48) × (1.5–)2–3(–3.5) µm; proliferating sympodially, forming a straight rachis with distinct scars; scars crowded near the apex of conidiogenous cells, slightly pigmented, <1 µm diam. Conidia (3–)4.5–5.5(–6.5) × (2–)2.5–3(–4) µm, solitary, aseptate, pale brown, obovate to obconical, finely verrucose, hilum conspicuous, 1 µm diam.

Cultural characteristics — Aerial mycelium spreading on MEA after 14 d at 24°C in the dark; flat, raised, dense, velvety, and with entire margin. Surface of the colony on MEA olivaceous-green, olivaceous-black in reverse, and often with a spreading citron-yellow pigment.

Host characteristics — On the fruit peels, the fungus showed flyspeck signs. Colonies lacked macroscopic dark mycelial mats, were circular or irregular, appeared as groupings (5–20 sclerotium-like bodies/mm^2) of tiny spheres (about 200 µm) that were classified as discrete speck (DS) mycelia type (Batzer et al. 2005).

Specimens examined: **China, Anhui Province**: Suzhou City, Dangshan County, 34°24′59″N 116°19′28″E, on fruit surface of pear (*Pyrus pyrifolia* (Burm. f.) Nakai), Oct. 2012, WH Li LWHDSL1 (GenBank KC986369), LWHDSL5 (GenBank KC986370), LWHDSL26 (GenBank KC986371). **Jiangsu Province**: Xuzhou City, Suining County, 33°59′46″N 117°44′31″E, on fruit surface of apple (*Malus domestica* Borkh.), Oct. 2012, L Wang & YM Zhao WLXZSN33 (GenBank KC986372), WLXZSN43 (GenBank KC986373).

Discussion

Ramichloridium species have various morphological characteristics and lifestyles, such as human pathogens, plant pathogens, and saprophytes

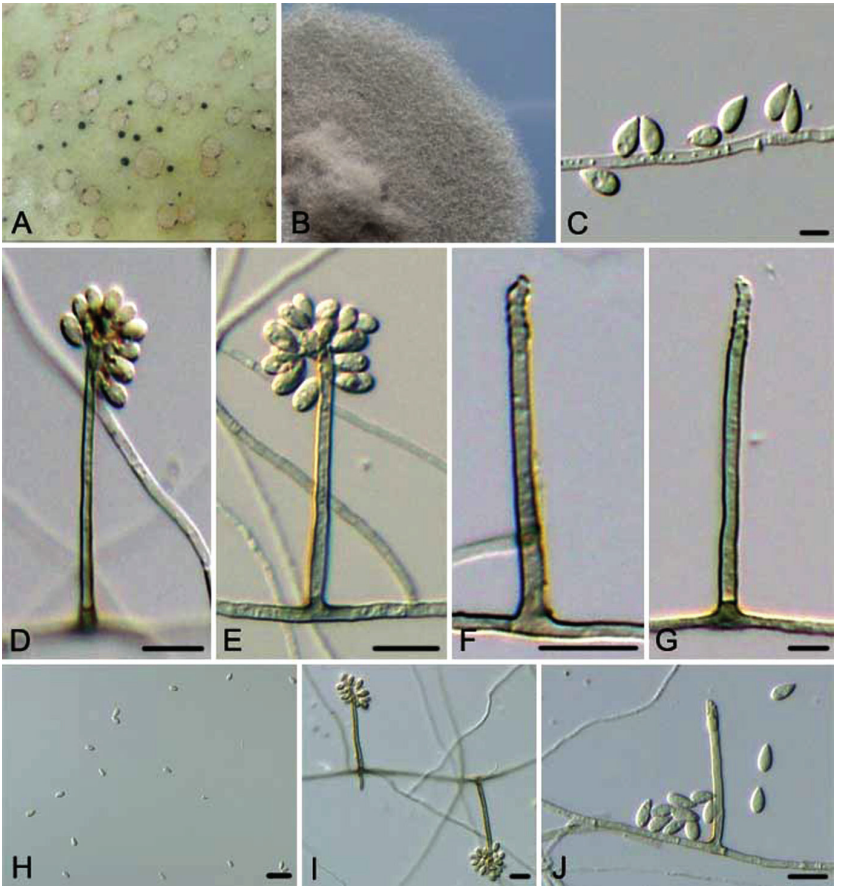

Fig. 2. *Ramichloridium apiculatum* (WLXZSN43). A. Discrete speck signs on pear; B. Colony on PDA; C, H. Conidia; D, E, I, J. Conidia and conidiogenous cells; F, G. Conidiophores. Scale bars: C, G = 5 μm; D–F, I, J = 10 μm; H = 20 μm.

(Arzanlou et al. 2007). *Ramichloridium apiculatum* was originally found in strongly deteriorated materials, in soil, and as a culture contaminant (de Hoog et al. 1977).

Among the pathogens commonly causing SBFS worldwide. *R. cucurbitae*, *R. punctatum,* and three other putative *Ramichloridium* species have been identified within the SBFS complex in the United States (Díaz Arias et al. 2010, Gleason et al. 2011, Li et al. 2012), while *R. luteum* (Li et al. 2012), *Dissoconium mali* (≡ *Ramichloridium mali*) (Zhang et al. 2007), and *R. streliziae* (Hao et al. 2013) are previously reported SBFS pathogens in China. This study, which

represents the first record of this species from China, provides the first evidence that *R. apiculatum* can be a causal agent of sooty blotch and flyspeck on apple and pear.

Acknowledgments

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